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<p>(21) International Application Number: PCT/GB88/00738 (22) International Filing Date: 7 September 1988 (07.09.88)</p> <p>(31) Priority Application Number: 8721015 (32) Priority Date: 7 September 1987 (07.09.87) (33) Priority Country: GB</p> <p>(71) Applicant (<i>for all designated States except US</i>): AMER-SHAM INTERNATIONAL PLC [GB/GB]; Amer-sham Place, Little Chalfont, Buckinghamshire HP7 9NA (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): McKAY, Ian Alexander [GB/GB]; 17 Sarre Road, London NW2 3SN (GB). MORSE, John [GB/GB]; 25 Woodside Close, Amersham, Bucks. HP6 5EF (GB). McFARTHING, Kevin, Gerard [GB/GB]; 34 School Lane, Weston Turville, Aylesbury (GB). GLOVER, Richard, Peter [GB/GB]; 23 Kerrison Road, Ealing, London W5 5NW (GB).</p>		<p>(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 5 Quality Court, Chancery Lane, London WC2A 1HZ (GB).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: MODIFYING LIVING CELLS</p> <p>(57) Abstract</p> <p>A method of introducing material into living mammalian cells, or of fusing material with the cells, comprises subjecting the cells in liquid suspension in the presence of the material to ultrasonic excitation sufficient to traumatisate the cells. The material introduced into the cells, or into a cell membrane, is preferably DNA or RNA or a protein.</p>			

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MODIFYING LIVING CELLS

This invention concerns the use of ultrasound to
5 introduce material into, or fuse material with, living
mammalian cells. The technique involves traumatising
the cells, but not killing or disintegrating them.
Ultrasound involves mechanical vibration at frequencies
generally too high for the ear to detect, generally
10 from 18kHz to 20MHz.

Ultrasound has been used for diagnostic and
therapeutic purposes on living mammalian tissue. The
maximum intensity that can be applied without hazard
has been reviewed by W.D.Ulrich (IEEE Transactions on
15 Biomedical Engineering, January 1974, pages 48 to 51).

Ultrasound is widely used to decompose or
disintegrate complex chemical species, such as polymers
including DNA. Ultrasonic disintegrators for this
purpose are commercially available.

20 Scanning acoustic microscopes use low intensity
vibrations in the MHz to GHz range.

A variety of methods of introducing material into
living cells exist, including calcium phosphate
precipitation and electroporation. Electroporation
25 involves the exposure of cells to a pulsed electric
field which presumably creates pores in the plasma
membrane. It has been used to introduce DNA into both
plant and animal cells, and has been successfully
applied to a wide range of cell types which have not
30 been accessible to other methods (G.Chu et al. Nucleic
Acids Research, Volume 15 number 3 1987, pages 1311 to
1326).

Similarly, a variety of methods exists for fusing
cells, including natural methods involving viruses such
35 as Sendai or HIV, and artificial methods involving

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polyethylene glycol mediated fusion or electrofusion. The electrofusion technique comprises two main processes, dielectrophoresis, by which a close contact between cells is established, and electrical membrane breakdown which gives rise to small pores in the cell membranes resulting in fusion of two apposed membranes in close contact. (K.Ohnishi, Journal of Immunological Methods, 100 (1987) 181 to 189.

Ultrasonic forces may be used to achieve the desired close contact between cells. Ultrasound wavelengths may be used that are much smaller than the fusion chamber. This permits not only the production of pearl chains of cells (in a purely propagating wave) but also the concentration of cells at standing-wave pressure maxima. W.M.Arnold et al. (Biochemical Society Transactions, 1986, pages 246 to 249) used 1.0 MHz ultrasound (1mm wavelength) to concentrate erythrocytes or myeloma cells, for fusion by a high voltage pulse.

The present invention provides a method of introducing material into, or fusing material with, living mammalian cells, which method comprises subjecting the cells in a vessel, in the presence of the material or immediately prior to the addition of the material, to ultrasonic excitation sufficient to traumatise the cells.

Briefly cells in suspension are exposed to ultrasound frequencies in the KHz to MHz range. These frequencies can induce oscillations in the cells, or cavitation in the vicinity of the cells. The resulting stresses in the cell membranes can allow total disruption of the cell, pore formation in the cell membrane or fusion of two (or more) closely apposed cells. For the duration of this cell disruption, substances in the solution in which the

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cells are suspended may be incorporated into those cells.

The cells are subjected to ultrasonic excitation sufficient to traumatisise them. This implies that the 5 cells are altered sufficient to effect entry of apposed material and, may be temporarily damaged, but not killed or disintegrated. Probably, pores are momentarily formed in the cell membrane, enabling apposed material to enter the cells or fuse with them.

10 In generating sufficient ultrasound intensity to traumatisise the cells, it may be unavoidable that some of them are killed.

The nature of the mammalian cells is not critical. The cells are preferably maintained in suspension in 15 aqueous or other fluid, but may alternatively be treated while adhering to a support.

The ultrasound intensity is chosen to be sufficient to traumatisise the cells but without permanently damaging or killing them. Suitable 20 intensities depend on a large number of factors, and are readily determined empirically for a particular experimental set-up. The ultrasound frequency is generally chosen in the range 18kHz to 20MHz. The time of treatment may be chosen empirically, but should 25 not be so long that an unacceptable temperature rise occurs. Treatment may be continuous or in pulses. Total treatment times of a few seconds to a few minutes are likely to be appropriate.

Materials which may be introduced into living 30 cells by this technique include, proteins, nucleic acids, oligonucleotides, DNA, lipids and lipid vesicles. Cells which take up these materials during ultrasonic excitation can survive the process and replicate subsequently. Genes introduced into cells 35 by this technique can be expressed and can be

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transmitted to daughter cells in a heritable fashion.

Instead of being introduced into the cell 5 interior, materials may be incorporated by this technique into the cell membrane. Examples of materials for this purpose include lipids, hydrophobic proteins, membrane receptors, lipid vesicles and liposomes. Furthermore, ultrasonic excitation can be 10 used to induce fusion of two or more apposed similar or dissimilar cells to form a single cell.

The cells are preferably maintained in suspension in an aqueous or other liquid medium. Cell 15 concentrations are generally in the range 10^4 to 10^8 cells/ml. Although the material is preferably present at the time of ultrasonic excitation, it may under some circumstances be introduced during or after the excitation while the cells remain traumatised. Preferably, the concentration of the other material 20 should also be kept at a high level. As discussed below, it is possible to use ultrasonic or other techniques to achieve localised high concentration, both of cells and of other material, in a suspension.

Various expedients, known to improve efficiency in 25 the prior art processes of electroporation and electrofusion, are also applicable in the present invention. Adjuvants may be present, including glucose/CaCl₂/MgCl₂, polyethylene glycol, albumin, calmodulin, phosphatidylserine, glycerylmono-oleate, 30 cholesterol, for cell fusion. The pH, salt concentration and temperature of the suspension are all factors which may affect efficiency. For transfecting DNA into cells, the presence of carrier DNA, such as sonicated salmon sperm DNA, may increase 35 efficiency.

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The cells are held in a vessel, whose size and shape and material of construction (acoustic impedance) need to be chosen in relation to the sonicating equipment being used. Small (bijou) polystyrene

5. vials or multi-well tissue culture plates are suitable. Ultrasonic excitation may be provided by one or several ultrasonic transducers, which may be positioned above, beside or below the vessel, or may have a probe which dips into the liquid suspension in the vessel. Or
- 10 the liquid suspension may be caused to flow in a controlled manner past one or more ultrasonic transducers.

Ultrasonic or other means may be provided for concentrating the cells at particular regions (e.g.

15 standing waves) in the liquid suspension. One ultrasonic transducer may be provided to concentrate the cells, while another is operated periodically to traumatise them. Or the same transducer may be used for both purposes, with the power setting being 20 periodically switched from a low level to a higher one. Control of the ultrasound frequency or frequency spectrum may be used to optimise the fusion of cells or uptake of material.

The following Examples illustrate the invention.

25 Example 1

Equipment

Soniprep 150 Ultrasonic Disintegrator - (MSE Ltd.,
Crawley)

Polystyrene bijou vials

30 9cm tissue culture petri dishes

Cells

63. Ag8.653 mouse myeloma cells suspended in DMEM with

10% calf serum. DMEM is Dubbecco's modified eagles

35 medium.

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DNA

pSV2 neo/EcoR1 digest (Southern P., and Berg P., (1982) J. Mol. Appl. Genet., Vol 1, p.327). Mouse carrier DNA: high molecular weight DNA isolated from 5 Balb/c mouse spleens and livers and partially sheared to reduce overall molecular weight.

Method

Three amplitude settings were tested for effects on cell viability.

10	Setting	Approximate % Survival after
		10 second pulse
	0	100
	1	90
	3	10

15

The cells were sonicated at the same settings in three separate bijou (5×10^6 cells in 5ml DMEM/bijou) for 10 seconds in the presence of 20ug pSV2 neo/EcoR1 digest and 50ug mouse carrier DNA.

20 After sonication, the contents of each bijou were transferred to 6cm petri-dishes and cultured for two days at 37°C on standard tissue culture conditions in an atmosphere of 100% humidity, 90% air and 10% CO₂.

25 The cells were then transferred to large vessels thus

Setting 0	3 x 9cm petri-dishes
Setting 1	3 x 9cm petri-dishes
Setting 3	1 x 9cm petri-dishes

30 To each dish fresh DMEM + 10% calf serum was added to make the volume 10ml. The antibiotic G418 was then added to a concentration of 1mg/ml medium. The cells were then incubated for five days before being transferred to larger vessels. Inspection of the 35 petri-dishes before this last transfer revealed

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numerous surviving/replicating myeloma cells in the setting 3 dish. Fewer survivors were seen in the setting 1 dish and none were seen in the setting 0 dish. Therefore the pSV2 neo plasmid DNA had 5 entered the sonicated myeloma cells (but not the control cells) and had integrated into those cells in a heritable fashion. This plasmid conferred resistance to the antibiotic G418 on the sonicated cells. The sonicated cells therefore survived in the 10 antibiotic whereas the control cells, which received no plasmid did not.

After a further two weeks the cells still survived in the antibiotic, indicating that the neo gene was integrated into the cells in an heritable fashion.

15 Example 2

Sonification of DNA into a Fibroblastic Cell Type

Equipment

Soniprep 150 Ultrasonic Disintegrator - MSE

Linbro 24 well tissue culture plate - Flow

20 Laboratories

9cm tissue culture petri-dishes - NUNC

Cells

Psi-2 fibroblasts - a retrovirus packaging line
(Ref:- Cepko, C.L. et al., Cell 37, 1053-1062, 1984).

25 DNA

ZNR3 plasmid consisting of a cDNA encoding the human N-ras protein inserted at the Bam H1 site of the pZIP SV(X) plasmid (op. cit.)

30 Mouse carrier DNA: high molecular weight DNA isolated from Balb/c mouse spleens and livers and partially sheared to reduce overall molecular weight.

Method

Psi-2 cells were introduced into the wells of the Linbro plate in suspension at 9×10^5 cells/ml medium 35 (DMEM - Flow Laboratories with 10% donar calf bovine serum - Flow Laboratories).

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As in Example 1, the tip of the exponential microprobe of the MSE Soniprep 150 was placed in the centre of the well, in this case at a depth of 1mm below the surface of the medium. Power was applied to 5 the microprobe for 10 seconds in each case. The effects of three different "amplitudes" (as read from the scale on the MSE apparatus) were tested on cell viability:-

10	Setting	Viable Cells
	0	8.7×10^5
	1	5.4×10^5
	3	1.9×10^5

15 For the actual experiment two wells were treated at each setting. In each well, in addition to 9×10^5 cells (initially) 10ug ZNR3 DNA and 10ug mouse-carrier DNA were included. After sonication for 10 seconds at each setting 0.2ml of cell suspension was withdrawn 20 from each well for counting. Cell counts were as above (i.e. DNA did not alter cell viability upon sonication in this experiment).

The remaining cells were left to attach to the bottoms of the wells in which they were contained 25 37°C in an atmosphere of 100% humidity, 9% CO₂/91% air.

After two days (sonication day 0 - cell splitting day 2) the cells were detached from the bottoms of the wells using a trypsin/EDTA solution (Flow Laboratories 30 2ml/well).

The cells were transferred to 2 x 10cm tissue culture petri dishes/well, i.e. 4 petri dishes/setting of the sonicator.

9ml fresh medium were added to each petri dish 35 which were then returned to the 37°C incubator and

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incubated as before. The medium in this case, however, contains 1mg/ml G418. Cells which had taken up the plasmid ZNR3 were resistant to the antibiotic as the plasmid contained the same gene as that found in 5 the pSV2 neo-plasmid of Example 1. Medium was changed on day 18. On day 20 the number of colonies of G418 resistant Psi-2 cells on the petri dishes (a colony was considered a group of greater than 16 cells) was counted.

10 There results were as follows:-

Setting	No. of colonies/4 petri dishes
0	0
1	207
15	0

This gives an efficiency of 10 colonies/ 10^6 cells surviving sonication/10ug plasmid DNA or 1/ 10^5 cells/ug.

20 Different cell types may need different sonicator settings and different adjuvants to achieve maximum efficiency.

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CLAIMS

1. A method of introducing material into, or fusing material with, living mammalian cells, which method comprises subjecting the cells in a vessel, in the presence of the material or immediately prior to addition of the material, to ultrasonic excitation sufficient to traumatise the cells.
2. A method as claimed in claim 1, wherein the material introduced into the cells is DNA, or RNA or protein.
3. A method as claimed in claim 2, wherein the material introduced into the cells is a gene, and the cells are subsequently caused to replicate so that the gene is expressed and transmitted to daughter cells in a heritable manner.
4. A method as claimed in claim 1, wherein the material is incorporated into the cell membrane.
5. A method as claimed in claim 1, wherein a mixture of two or more similar or dissimilar cell types is subjected to the ultrasonic excitation to induce fusion of two or more cells.
6. A method as claimed in any preceding claim, wherein the cells are maintained in suspension in a liquid.
7. A method as claimed in claim 6, wherein the cells are concentrated in suspension by means of ultrasonically induced standing waves.
8. A method as claimed in any one of claims 1 to 7, wherein, during and after ultrasonic excitation, the cells are maintained in a suitable form for reintroduction into a host mammal.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 88/00738

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁴

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00; C 12 N 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC ⁴	C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemical Abstracts, volume 72, 27 April 1970, (Columbus, Ohio, US), A.Sh. Lazaretnik et al.: "Phono-phoresis in conditions of experimental tumors and leucoses", see page 187, abstract 88206s, & Fiziol. Zh. (Kiev) 1969, 15(6), 842-6 --	1
A	Chemical Abstracts, volume 104, 7 July 1986, (Columbus, Ohio, US), V.I. Svidovyi et al.: "Changes in aminotransferase activity and the permeability of erythrocyte membranes in relation to ultrasound and low frequency noise", see page 442, abstract 86056y, & Gig. Sanit. 1985, (10), 73-4 --	1
A	Chemical Abstracts, volume 104, 7 April 1986, (Columbus, Ohio, US), T.N. Julian: "The effects of ultrasound on drug permeation through .".	

* Special categories of cited documents: ¹⁰

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

29th November 1988

Date of Mailing of this International Search Report

19 DEC 1988

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer


P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	<p>membrane barriers", see page 393, abstract 116006b, & Diss. Abstr. Int. B 1985 46(2), 532 --</p>	
A	<p>DE, A, 3505161 (GCA CORP.) 21 August 1986 --</p> <p>Biological Abstracts, volume 78, no. 9, 1984, (Philadelphia, PA., US), V.V. Kuklin et al.: "Using the method of protoplast fusion in the selection of <i>Streptomyces</i> <i>griseus</i> producing grinin, a strepto- thricin antibiotic", see page 7520, abstract 66698, & Antibiotiki (Mosc) 28(12): 883-889, 1983</p> <p>-----</p>	

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 8800738
SA 24184

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/12/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A- 3505161	21-08-86	None	